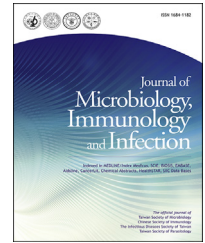




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Original Article

# Immunologic evaluation of extracted intestinal proteins from *Angiostrongylus cantonensis* adult worms



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## KEYWORDS

*Angiostrongylus cantonensis*;  
Intestinal proteins;  
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**Abstract** *Background:* To determine whether intestinal *Angiostrongylus cantonensis* antigens can induce protective immunity in rats, gut antigens prepared from female adults (FAGP) and somatic antigens prepared from both male (MA) and female (FA) adult worms were used to immunize rats.

*Methods:* Rats were immunized twice with MA, FA, or FAGP antigens and then challenged with 50 third-stage *A. cantonensis* larvae, and different readouts were used to monitor protective immunity. Additionally, protein profiles of MA, FA, and FAGP extracts were analyzed and characterized by immunodetection methods.

*Results:* A 15% reduction in fifth-stage larvae from brains and a 14% reduction in adult worms from pulmonary arteries were observed in rats immunized with FAGP compared to controls. However, there was a >50% reduction in rats immunized with MA or FA. The lengths of larvae and adults recovered from FAGP-immunized rats were shorter than those recovered from other groups. The number of first-stage larvae recovered from fecal material in FAGP-immunized rats was significantly reduced. Additionally, FAGP induced the highest splenocyte proliferation.

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Serum IgG titers were not directly correlated with protective immunity. An 84 kDa gut membrane protein was strongly recognized by anti-FAGP antibodies.

**Conclusion:** We demonstrated that immune responses induced by FAGP reduced the growth, development, and reproduction of *A. cantonensis* in subsequent infections. While the possibility of using FAGP combining with MA or FA antigens as a multi-function vaccine in immune protection against *A. cantonensis* needs to be further elucidated, we hope that it provides a novel strategy for this parasite vaccine development.

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## Introduction

*Angiostrongylus cantonensis* is a parasite that mainly resides in rat heart and pulmonary arteries, and in humans causes eosinophilic meningitis and meningoencephalitis in the Far East, Southeast Asia, and Pacific Islands.<sup>1–3</sup> Many terrestrial and freshwater mollusks, such as *Achatina fulica* and *Ampullaria canaliculata*, can act as intermediate hosts, which spread this parasite in Taiwan. When mammals ingest these contaminated intermediate hosts, they can become infected.<sup>4–6</sup> These larvae migrated to the brain of infected individuals cause brain and spinal cord symptoms, such as headache, fever, vomiting, lethargy, stiff neck, and increased cerebrospinal fluid (CSF) pressure.<sup>3,7</sup>

*A. cantonensis* remains an important zoonotic parasite to local communities in Taiwan and is endemic in parts of China.<sup>1,3,8,9</sup> Presently, there is still no effective treatment for this parasite. Acquired immunity against *A. cantonensis* has been studied in experimental rodent hosts treated with various types of parasite antigens, such as crude somatic antigenic extracts from larvae of different developmental stages,<sup>10–13</sup> excretory-secretory (ES) antigens prepared from adults,<sup>14,15</sup> gamma-irradiated third-stage larvae<sup>16</sup> and live third-stage larvae.<sup>11</sup> Importantly, studies have reported that rats infected with *A. cantonensis* can develop acquired immunity against re-infection with the same parasite species.<sup>16,17</sup> Antibodies in rats infected with *A. cantonensis* increase significantly in the serum and CSF where they associate with eosinophils to induce subsequent immune responses against this parasite.<sup>18,19</sup> Yong et al. also found that passive transfer of immune serum or lymphocytes from infected rats to the uninfected recipient produced protective immunity against the same infection.<sup>20,21</sup>

Animals immunized with soluble or membrane associated proteins extracted from the gut of hematophagous parasites were shown to be protected against infection.<sup>22–25</sup> For example, intestinal enzymes as well as integral membrane proteins from *Haemonchus contortus* were shown to protect lamb hosts from nematode infection.<sup>26–28</sup> The principal candidate antigens that are expressed in the gut of these parasites generally show protease activity, and include aspartyl protease, an integral membrane glycoprotein complex located on intestinal cells. Antibodies are thought to mediate protective immunity by blocking the activity of such enzymes that are involved in digestion within parasitic worms.<sup>29,30</sup> Accordingly, the present investigation describes a valuation of gut

proteins extracted from female *A. cantonensis* adults in the protection of rats against challenge infection, and provides an assessment of the acquired immunity in rats treated with these proteins compared to those treated with somatic antigens extracted from male and female adults.

## Materials and methods

### Animals and parasites

Sprague–Dawley (SD) rats used in this study were purchased from the National Laboratory Animal Center (NLAC), Taipei, Taiwan. The Taiwan strain of *A. cantonensis* was originally isolated from *A. canaliculata*, collected from the Yen-Tsao district, Kaohsiung, Taiwan. This strain was maintained in the laboratory by cycling through *Biomphalaria glabrata* snail and SD rats. First-stage larvae (L1) were recovered from the feces of infected rats and fed to snails. Infective third-stage larvae (L3) were obtained from the snails after artificial digestion with 0.6% pepsin–HCl (pH 2.5). Rats were infected via stomach intubations. Animal experiments were carried out under humane conditions with approval (LAC2014-0203) from the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University.

### Antigen preparations

Adult *A. cantonensis* worms were obtained from the pulmonary arteries and hearts of infected rats. These worms were homogenized in 0.15 M PBS (pH 7.4) in a glass tissue grinder at 4 °C, and sonicated in an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments, Manor Royal, UK). Somatic antigens were prepared from male adults (MA) and female adults (FA) by centrifugation at 12,500 g for 45 min at 4 °C. Soluble extracts of FA gut proteins (FAGP) and gut membrane proteins were prepared following previous report with modified protocol.<sup>24</sup> Briefly, some female worms were dissected, and intact intestines were carefully collected under a dissecting microscope, and then gently squeezed to remove out all the material inside. These intestines were washed with 0.15 M PBS (pH 7.4) for several times, and then disrupted for 10 min homogenization and following sonication and centrifugation as described above. In addition, insoluble gut samples (1 mg of proteins) were homogenized in the homogeneous liquid (1 mM

ethylenediamine-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.15 M PBS) to 10% (w/v) lysate. Homogenized lysate was centrifuged at 12,500 g for 10 min at 4 °C, and added 0.1% Tween 20, and then centrifuged again. After removing supernatants, homogeneous liquid with 2% Triton X-100 were added and placed for 2 h at 4 °C, and then centrifuged again. All supernatants of samples were stored in aliquots at -70 °C. The protein concentration of each antigen extract was determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Richmond, CA).

### Immunization and challenge infection

Totally sixty female SD rats (eight-week-old) were divided into four groups that received either MA, FA, or FAGP antigens, or PBS as a control. The immunization procedure is modified and referenced concurrently according to the previous articles.<sup>11,24</sup> For the first immunization, each rat was injected subcutaneously with 1 mg of antigen extract emulsified in Freund's complete adjuvant (FCA). Two weeks later, each rat received a booster injection with 500 µg of antigen extract emulsified in Freund's incomplete adjuvant (FIA). Control rats received two injections of PBS emulsified first in FCA and then in FIA. Three days after the last immunization, each rat received an oral challenge infection via stomach intubation of 50 active *A. cantonensis* L3. After twice immunizing and challenging, three rats of every group were euthanized and their serum and spleens collected for detection.

### Parasitological techniques

Three rats in each group were euthanized 19 days after the challenge infection. The brains, hearts, and lungs were examined at necropsy, and different stages of worms were collected and their lengths measured carefully. The feces of other rats were collected every day from the 35th day after infection, and the numbers of L1 that appeared in the feces of each rat were carefully monitored until the 50th day. The harvest and counting for the number of L1 of *A. cantonensis* were according to the previous references with modifying.<sup>31,32</sup> Then all rats ( $N = 3$  in each group) were euthanized, and their brains, hearts, and lungs were examined, the numbers and lengths of parasite were carefully counted and measured.

### Cell proliferation assay

The splenocytes collected were analyzed with the Biotrak cell proliferation ELISA system version 2 (Amersham Pharmacia Biotech; APB, Piscataway, NJ). The splenocytes were cultured in 96-well plates ( $1 \times 10^5$  cells/well) with 200 µl RPMI-1640 culture medium (CM). The cells were stimulated with 6 µg of antigen or 2 µg of concanavalin A (Con A) and incubated for 24 h at 37 °C. Next, 20 µl 5-bromo-2'-deoxyuridine (BrdU) labeling solution were added and cultured for 6 h. Subsequently, the cells were centrifuged at 2000 g for 20 min and then dried for 60 min at 60 °C. Each well was fixed with 200 µl of FixDenat solution for 30 min. After washing, 100 µl of a 1:100 dilution of a peroxidase-conjugated mouse anti-BrdU mAb was incubated for

60 min at 37 °C. The substrate solution was added and stopped 30 min later with 25 µl of 1 M H<sub>2</sub>SO<sub>4</sub>. The stimulation index (SI) of each group was calculated as the mean of the OD<sub>450</sub> values from triplicate antigen- or Con A-treated cultures divided by the mean of the OD<sub>450</sub> values from triplicate CM-treated cultures.

### Antibody assay and western blotting

The capability of FAGP and FA antigens to induce specific antibodies was evaluated by an enzyme-linked immunosorbent assay (ELISA). FAGP or FA antigens were incubated overnight at 4 °C (20 µg protein/well). After emptied, the wells were blocked with 0.5% non-fat skim milk for 30 min at 37 °C. After blocking, a 1:2000 dilution of sera was added and incubated for 1 h at 37 °C. After four washes, a 1:500 dilution of affinity purified horseradish peroxidase (HRP)-goat anti-rat IgG was added and incubated for 30 min at 37 °C. Then, each well then received 100 µl of 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) peroxidase substrate solution (Zymed, San Francisco, CA). The optical density was read after 30 min at RT by a Dynatech MR5000 microplate reader (Dynatech, Germantown, MD). Western blot analysis was conducted to determine the components of antigens. Aliquots containing 100 µg/ml of the antigens were resolved by 12% homologous SDS-PAGE, and then electrophoretically transferred to polyvinylidene difluoride (PVDF, Millipore, Billerica, MA) membranes. Strips of the membranes were cut and blocked and then incubated with rat sera for 60 min at 37 °C. After washing, the strips were incubated with HRP-goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 35 min at 37 °C. After washing, a tetramethylbenzidine (TMB) substrate solution (Thermo-Fisher Scientific, Waltham, MA) was added for staining.

### In situ antigen localization in the worm

An indirect immunofluorescence assay (IFA) was performed to evaluate the distribution of these reactive gut antigens. The adult worms were dehydrated through graded alcohols, embedded in paraffin, sliced into thin sections, and mounted onto slides following standard procedures for paraffin section preparation. The slides were deparaffinized with xylene, fixed with acetone, and incubated with sera collected from FAGP-immunized rats for 1 h at 37 °C. After washing, rhodamine-labeled goat anti-rat IgG was applied to the slides. The slides were incubated in a moist chamber for 30 min at 37 °C, and then washed with PBS. One drop of 70% glycerol and a coverslip were then added to each slide. Finally, the slides were examined with a fluorescence microscope (Olympus, Tokyo, Japan).

### Statistical analysis

Antibody levels were compared by one-way ANOVA using SPSS 18.0 software (SPSS Inc. Chicago, IL, USA). Other results were analyzed using two-tailed Student's *t*-tests. In all analyses, *p* values < 0.05 were considered significant, and data are expressed as the means ± standard deviations (SD).

**Table 1** Protective effects against L5 in the brains and adult worms in the hearts and pulmonary arteries of rats immunized with different *A. cantonensis* antigen preparations.

Group	Worms recovered (mean $\pm$ SD)			% Protection
	Male	Female	Total	
<b>L5</b>				
MA	3.5 $\pm$ 2.12*	5 $\pm$ 0.00*	8.5	69
FA	6.5 $\pm$ 0.71*	4.5 $\pm$ 0.71*	11.0	60
FAGP	11.0 $\pm$ 1.41	12.5 $\pm$ 0.71*	23.5	15
Challenge	11.5 $\pm$ 0.71	16 $\pm$ 2.83	27.5	–
<b>Adults</b>				
MA	6.0 $\pm$ 4.24*	8.5 $\pm$ 0.71*	14.5	50
FA	6.5 $\pm$ 3.54*	7.5 $\pm$ 3.54*	14.0	52
FAGP	12.5 $\pm$ 3.54	12.5 $\pm$ 0.71	25.0	14
Challenge	14.0 $\pm$ 1.41	15.0 $\pm$ 4.24	29.0	–

Each rat was challenged with 50 L3 ( $N = 3$  in each group). Tests of significance (Student's *t*-tests) were performed between each group and the challenge control, \* $p < 0.05$ .

## Results

### Evaluation of the protective effect of FAGP immunization

To test the protective effect of FAGP against *A. cantonensis* infection, different immunized groups of rats were challenged with 50 *A. cantonensis* larvae, and recovered worms were counted at 19 and 50 days post-infection. Table 1 shows fifth-stage larvae (L5) recovered from the brains and adult worms recovered from the hearts and lungs of rats with different antigen immunizations. Compared to the control group in the challenge infection, significantly less female L5 were recovered from rats in all three immunized groups, while there were less male L5 recovered only in the MA and FA immunized groups ( $p < 0.05$ ). Worm reduction rates of 69%, 60%, and 15% were achieved in rats immunized with MA, FA, and FAGP, respectively. Likewise, reductions of 50%, 52%, and 14% in adult worms recovered from the hearts and pulmonary arteries were observed in rats immunized with MA, FA, and FAGP, respectively. However, there were significant differences between MA and FA groups from controls ( $p < 0.05$ ), while the FAGP immunized group was not.

The lengths of adult worms recovered from rats immunized with antigens prepared from *A. cantonensis* were significantly shorter than those of worms recovered from the control groups (Table 2,  $p < 0.05$ ). On the contrary, the differences in the lengths of L5 recovered from the different groups were not significant. Although the reduction in the number of worms recovered from rats immunized with FAGP was not as significant as those in rats immunized with antigens extracted from whole MA or FA, the adult worms recovered from rats immunized with FAGP had the shortest lengths among all groups ( $p < 0.01$ ). Table 3 shows the population dynamics of *A. cantonensis* L1 from all immunized rats during the challenge infection. Our data indicate that the number of L1 recovered from fecal material dropped after 45 days post-infection in rats immunized with FA and FAGP ( $p < 0.05$ ), while there was only a dramatic and sustained reduction in worms recovered from 45 to 50 days following challenge infection in FAGP-immunized rats.

### Splenocyte proliferative responses in rats following immunization and infection with *A. cantonensis*

The proliferations of antigen-stimulated splenocytes from rats that received different antigens were analyzed, and the results are shown in Table 4. The SIs of rats immunized with FA, MA, and FAGP were significantly higher than that of rats in the control group ( $p < 0.05$ ). However, rats immunized with FAGP exhibited the highest SI values among all the antigen-immunized groups, and the SI values gradually increased with each successive immunization.

### Specific antibody responses in rats following immunization and infection with *A. cantonensis*

Fig. 1 shows all of the ELISA values of anti-FAGP IgGs (Fig. 1A) and anti-FA IgGs (Fig. 1B) of sera from rats immunized with different antigens. Our results show the ELISA values of sera from MA, FA, and FAGP immunized rats against either FAGP or FA antigens were both significantly higher than that of sera from control rats ( $p < 0.05$ ;  $p < 0.001$ ). Furthermore, the IgG antibody responses in rats immunized with FAGP were higher after one immunization, but significantly lower after two immunizations than the respective responses of FA immunized rats ( $p < 0.05$ ). Among the sera from different groups, sera collected from rats immunized twice with antigens prepared from FA

**Table 2** The lengths of L5 in the brains and adult worms in the hearts and pulmonary arteries of rats immunized with different *A. cantonensis* antigen preparations.

Group	Length of L5 recovered (mm) (mean $\pm$ SD)		Length of adults recovered (mm) (mean $\pm$ SD)	
	Male	Female	Male	Female
MA	5.87 $\pm$ 0.57	7.59 $\pm$ 0.42	14.81 $\pm$ 0.40**	19.55 $\pm$ 0.58*
FA	5.80 $\pm$ 0.43	7.00 $\pm$ 0.16	15.54 $\pm$ 0.63*	19.38 $\pm$ 1.02*
FAGP	5.72 $\pm$ 0.23	6.99 $\pm$ 0.30	14.44 $\pm$ 0.66**	18.37 $\pm$ 0.85**
Challenge	5.78 $\pm$ 0.52	7.74 $\pm$ 0.85	15.82 $\pm$ 0.50	21.81 $\pm$ 1.93

Each rat was challenged with 50 L3 ( $N = 3$  in each group). Student's *t*-tests were performed between each group and the challenge control with \* $p < 0.05$  and \*\* $p < 0.01$ .



**Table 3** *A. cantonensis* L1 population dynamics following challenge infection of immunized rats in each group.

Day	Challenge	MA	FA	FAGP
<39	0	0	0	0
40	41.8 ± 13.2	12.5 ± 11.0	3.2 ± 1.8	24.7 ± 24.7
41	288.3 ± 23.7	79.0 ± 67.0	465.5 ± 324.5	471.4 ± 67.1
42	970.7 ± 656.1	253.3 ± 185.0	924.5 ± 652.6	881.9 ± 235.3
43	892.0 ± 411.9	466.2 ± 369.3	604.6 ± 286.4	1010.6 ± 121.4
44	902.6 ± 407.9	105.7 ± 14.5	992.3 ± 330.8	1071.4 ± 618.0
45	961.3 ± 378.2	838.2 ± 495.8	1546.7 ± 1013.3	581.8 ± 463.4*
46	1006.0 ± 122.0	685.0 ± 202.5	337.5 ± 212.5*	624.7 ± 40.3*
47	1769.4 ± 5.0	1404.8 ± 365.0	570.0 ± 380.0	782.6 ± 101.6**
48	1327.5 ± 654.2	1251.2 ± 148.8	2172.5 ± 1303.5	950.6 ± 33.4**
49	1357.9 ± 355.6	1637.4 ± 57.4	624.1 ± 37.8*	569.6 ± 67.5**
50	3062.7 ± 884.4	999.1 ± 125.3	2177.2 ± 1496.8	586.4 ± 24.3**

Student's *t*-tests were performed between each group and the challenge control with \**p* < 0.05 and \*\**p* < 0.01 (*N* = 3 in each group).

worms resulted in the highest ELISA values except those from normal infected rats. However, the responses of anti-FA IgGs were reached the highest in the sera from rats immunized twice with FA than others (*p* < 0.001). Similarly, the higher anti-FA responses also appeared in sera from rats immunized twice with FAGP than that immunized with once.

The somatic extracts of MA and FA worms were found to be highly complex, each of which consisted of more than 20 different polypeptides with molecular weights varying from 7.4 kDa to more than 205 kDa (Fig. 2). Fig. 2C shows that an 84 kDa protein present in all antigen preparations was clearly recognized by the immune serum elicited against FAGP antigens. Polypeptides with molecular weights of 21 and 22 kDa were present in all three antigen preparations (MA, FA and FAGP), but not in the intestinal membrane (lane 4, Fig. 2B and C), which may be representative of antigens present in the gut contents of FA worms.

#### Reactivity of anti-FAGP antibody from an immune rat to the *A. cantonensis*

Strong positive reactions were observed by indirect IFA in the internal musculature of the body, gut, and reproductive

tract walls as well as in the lumen of the gut of adult worms (Fig. 3).

#### Discussion

Rats immunized with somatic adult worm antigens can induce acquired immunity against *A. cantonensis* was previously demonstrated.<sup>11</sup> In the current study, we evaluated the acquired immunity against *A. cantonensis* infection after immunization of rats with FAGP, which was compared to that in rats immunized with somatic antigens extracted from MA and FA worms.

Immunizing rats with somatic adult worm antigens clearly enhanced their resistance to infection by reducing worm recovery more than 50%. Although worm recovery was not that dramatically reduced, worm development, growth and fecundity in FAGP-immunized rats were all reduced. Immature L5 recovered from the brains and mature worms recovered from the pulmonary arteries of rats immunized with FAGP were both stunted. Reduced L1 production by female adults in FAGP-immunized rats also indicated that the developmental and reproductive activities of *A. cantonensis* were suppressed in this group. Intestinal antigens from *H. contortus* induced protective

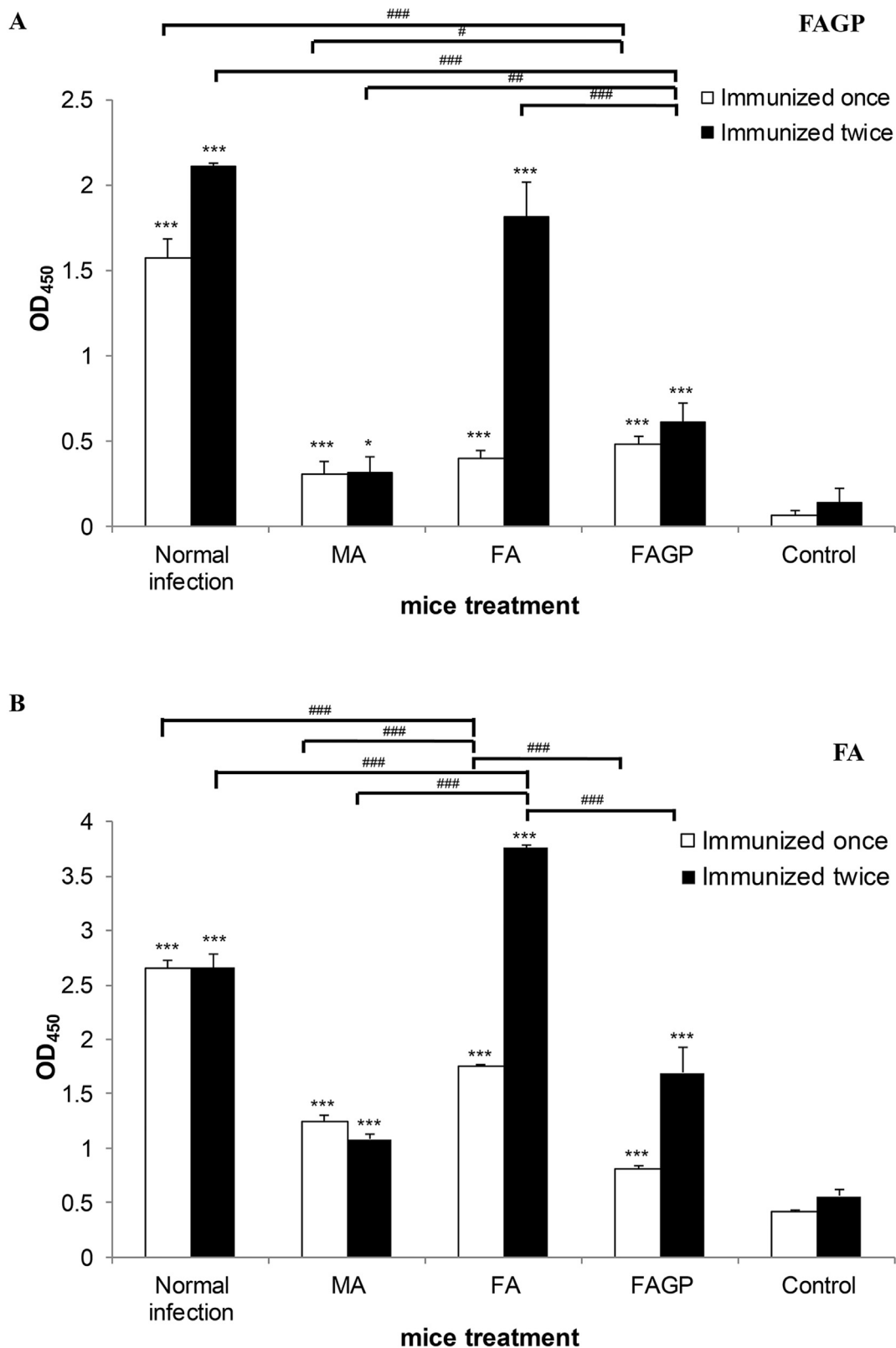
**Table 4** Proliferative responses of splenocytes from rats immunized with different *A. cantonensis* antigens preparations.

Group	SI <sup>a</sup>			
	Stimulator <sup>b</sup>	Before primary immunization	Before booster immunization	Before challenge infection
MA	MA	0.85 ± 0.40	1.24 ± 0.28*	1.05 ± 0.20*
	Con A	1.71 ± 0.13*	2.08 ± 0.08*	1.33 ± 0.10*
FA	FA	0.76 ± 0.24	1.15 ± 0.24*	1.19 ± 0.18*
	Con A	1.90 ± 0.14*	2.06 ± 0.46*	1.86 ± 0.37*
FAGP	FAGP	0.90 ± 0.19	1.05 ± 0.13*	1.39 ± 0.12*
	Con A	1.79 ± 0.13*	1.55 ± 0.11*	1.64 ± 0.15*
Challenge		0.87 ± 0.11		

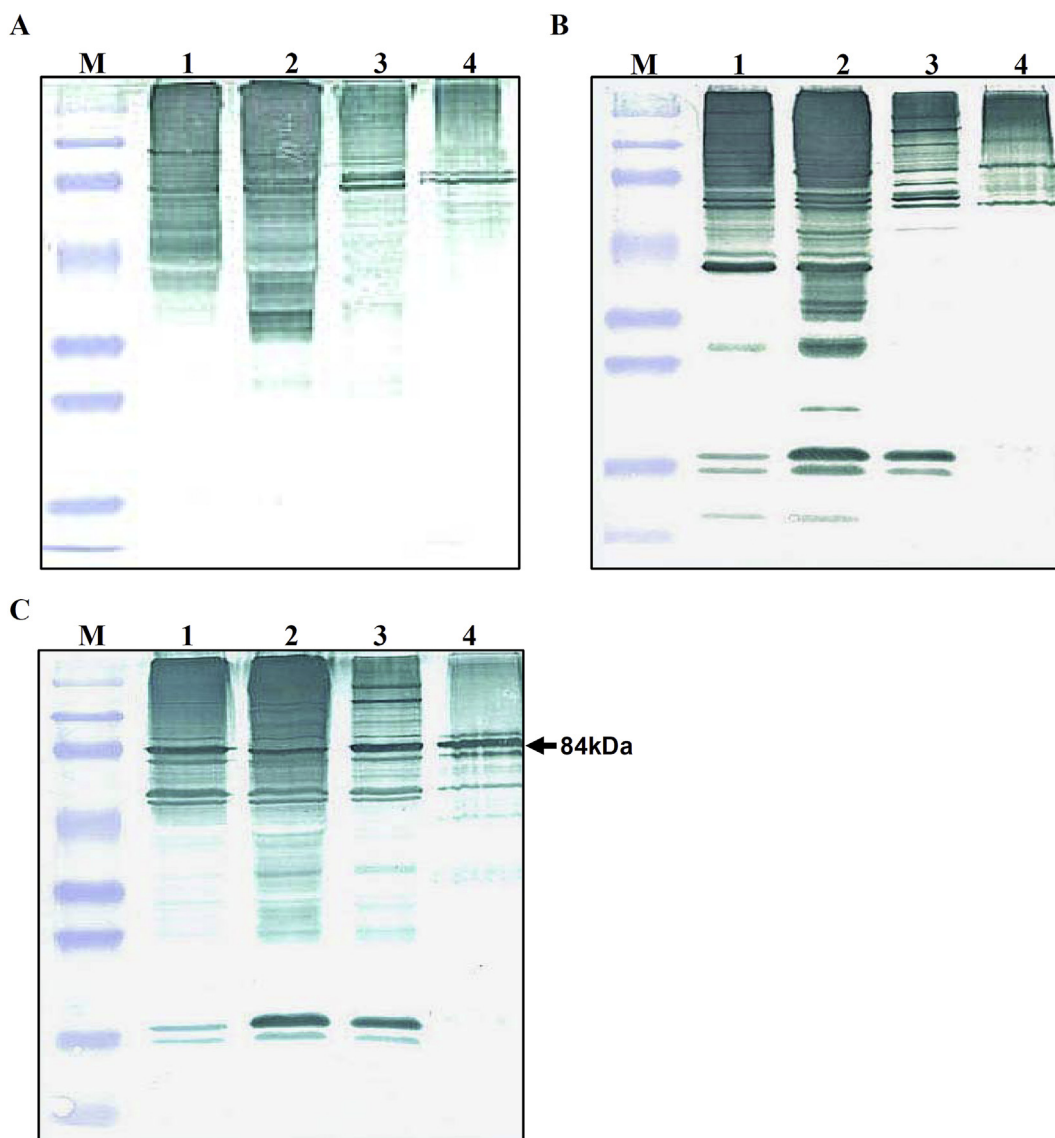
Student's *t*-tests were performed between each group and the challenge control with \**p* < 0.05 (*N* = 3 in each group).

<sup>a</sup> SI, stimulation index = mean OD<sub>450</sub> values from triplicate antigen-treated cultures/mean OD<sub>450</sub> values from triplicate CM-treated cultures.

<sup>b</sup> Splenocytes of rats from these groups were stimulated with 6 µg/well of antigen (MA) or 2 µg/well of Con A.



**Figure 1.** Antibody titers to antigens from (A) gut protein from female adults (FASP) and (B) female adults (FA) of *A. cantonensis* in rat sera determined by ELISA. Normal infection: positive control sera collected from rats 50 days after infection. MA, FA, and FASP: sera collected from rats immunized with antigens prepared from male adults, female adults, and gut protein from female adults, respectively. Control: negative control sera collected from rats immunized with PBS. Light bars indicate one immunization and dark bars indicate two immunizations. The results are shown as the means ± SD from three independent experiments. Number of samples was 3 in each group. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  indicate comparisons to the control group. # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  indicate comparisons between the two indicated groups.



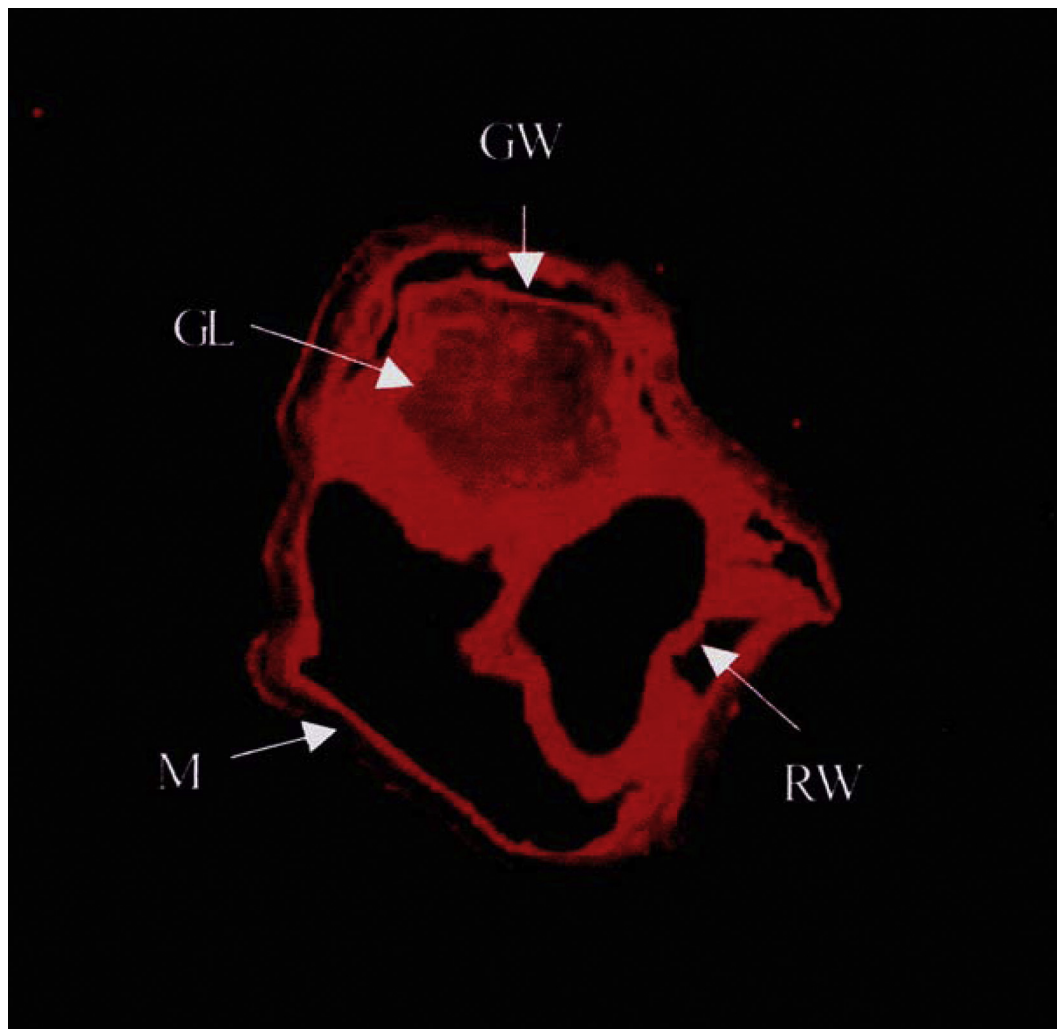
**Figure 2.** Western blot analyses of antigens prepared from *A. cantonensis* with sera from rats immunized with antigens prepared from (A) male adults (MA), (B) female adults (FA), and (C) gut protein from female adults (FAGP). Lanes 1–4, antigens prepared from MA, FA, FAGP, and gut membrane proteins, respectively. A standard protein marker is shown to the left with molecular weights of 7.4, 21.0, 30.2, 36.3, 52.2, 84.0, 120.0, and 205.0 kDa (lane M).

immunity in lambs resulting in a 65% reduction in worm recovery, 95% inhibition of egg production in the feces of lambs, and a reduction in worm growth so that recovered worms were much shorter.<sup>24,26,27</sup> The antifertility effects and impaired worm development of *A. cantonensis* by FAGP observed in this study are in agree with these reports on the *H. contortus*-lamb system.

Experimental infections of *A. cantonensis* in athymic, thymectomized or splenectomized animals are usually more severe.<sup>33</sup> Splenectomized rats exhibited decreased antibody responses while their cellular immune responses remained normal, and passive spleen cell transfers to these rats effectively restored their antibody responses.<sup>21</sup> Yong et al. showed that protective immunity to *A. cantonensis* infection was mainly humoral-mediated and could be passively transferred with immune serum.<sup>20</sup> Here we

focused on the proliferative responses of splenocytes from rats immunized with gut antigens. The SI values indicated that cell-mediated immune responses may not play a major role in *A. cantonensis* elimination, but indeed affected other factors such as worm growth and L1 production. Current report showed that *A. cantonensis* infection can cause splenic atrophy which is associated with a reduced proliferation of splenocyte and increased apoptosis.<sup>34</sup> It may be the reason that proliferative responses of splenocytes were not as significant as the antibody responses between both the primary immunization and booster.

Other study reported *A. cantonensis* infection induced Th2 immune response and the humoral immune responses play an important role on responding properly to parasite antigen and exert anti-parasite effects.<sup>35</sup> Antibodies from infected rat sera detected to antigens of various stages



**Figure 3.** Section of adult *A. cantonensis* recognized by rhodamine-labeled IgG antibody to gut membrane proteins by indirect IFA. M: internal musculature of the body. GL: gut lumen. GW: gut wall. RW: reproductive tract wall.

found to be more sensitive to adult than to larval antigens.<sup>36</sup> It was also shown that antibody responses were most pronounced during the oviposition of female worms and the time of lifting was consistent with that when L1 appear in the feces.<sup>37,38</sup> Our data showed that the anti-FAGP IgG titers of sera from rats immunized with antigens prepared from FA worms were the highest, and were even higher than those from FAGP-immunized rats. In addition, the anti-FA IgGs were reached the highest from rats immunized twice with FA, meanwhile, the trend of anti-FA IgGs in sera from rats immunized with FAGP were also similar as that. The results may be due to our FA extracted antigens also contained the constituents of FAGP.

Due to the similar composition, major proteins of the gut recognized by immune sera were also present in the gut membrane. An 84 kDa protein within the gut membrane may play a partial identified role in immune responses against *A. cantonensis* development and reproduction, because of the strong antigenic recognition by anti-FAGP antibodies observed herein. Vaccine studies with different gut membrane antigens from *H. contortus* have demonstrated their protective effects in lambs, and the most

effective gut antigens generally show protease activity involved in the digestion of blood meal.<sup>26–28,30</sup> This protective mechanism correlated with antibody immunity may be due to the inhibition of these enzymes by vaccine-induced antibodies, or the accumulation of antigen–antibody complexes on the intestinal surface that acts as a barrier to nutrient absorption.<sup>30,39</sup> In contrast, larval establishment is unaffected in sheep immunized with *H. contortus* gut membrane proteins, whereas egg output is severely reduced.<sup>27,30,40</sup> These observations are consistent with the results presented herein for *A. cantonensis*. In the indirect IFA, the identified worm bodies by anti-FAGP serum were mainly found on the intestinal lumen and outer edge of the gut wall; therefore, we presume effective antigenic gut proteins may be located in the intestinal membrane tissues of *A. cantonensis*.

The present study provides evidence indicating that immune responses against FAGP may reduce the growth, development, and reproduction of *A. cantonensis*. Due to the similarity in the antigenic composition, major proteins of the gut membrane recognized by immune sera are likely present in other kinds of membranes. Therefore, more



work is needed to understand the possibility of using intestinal membrane proteins combining with female adult worm somatic antigens as a multi-function vaccine in protective immunity against *A. cantonensis*. We hope that these data provide a novel strategy for the vaccine development aimed at interfering with parasite reproduction and survival.

## Conflicts of interest

The authors declare no conflicts of interest.

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## References

- Wang QP, Wu ZD, Wei J, Owen RL, Lun ZR. Human *Angiostrongylus cantonensis*: an update. *Eur J Clin Microbiol Infect Dis* 2012;31:389–95.
- Alicata JE. *Angiostrongylus cantonensis* and eosinophilic meningitis. *Lancet* 1988;2:1367–8.
- Wang QP, Lai DH, Zhu XQ, Chen XG, Lun ZR. Human angiostrongyliasis. *Lancet Infect Dis* 2008;8:621–30.
- Yang TB, Wu ZD, Lun ZR. The apple snail *Pomacea canaliculata*, a novel vector of the rat lungworm, *Angiostrongylus cantonensis*: its introduction, spread, and control in China. *Hawaii J Med Public Health* 2013;72:23–5.
- Chao D, Lin CC, Chen YA. Studies on growth and distribution of *Angiostrongylus cantonensis* larvae in *Ampullarium canaliculatus*. *Southeast Asian J Trop Med Public Health* 1987;18:248–52.
- Wang JJ, Chung LY, Lin RJ, Lee JD, Lin CW, Yen CM. Eosinophilic meningitis risk associated with raw *Ampullarium canaliculatus* snails consumption. *Kaohsiung J Med Sci* 2011;27:184–9.
- Yii CY. Clinical observations on eosinophilic meningitis and meningoencephalitis caused by *Angiostrongylus cantonensis* on Taiwan. *Am J Trop Med Hyg* 1976;25:233–49.
- Tsai HC, Chen YS, Yen CM. Human parasitic meningitis caused by *Angiostrongylus cantonensis* infection in Taiwan. *Hawaii J Med Public Health* 2013;72:26–7.
- Tseng YT, Tsai HC, Sy CL, Lee SS, Wann SR, Wang YH, et al. Clinical manifestations of eosinophilic meningitis caused by *Angiostrongylus cantonensis*: 18 years' experience in a medical center in southern Taiwan. *J Microbiol Immunol Infect* 2011;44:382–9.
- Dharmkrong-at A, Sirisinha S. Analysis of antigens from different developmental stages of *Angiostrongylus cantonensis*. *Southeast Asian J Trop Med Public Health* 1983;14:154–62.
- Wang LC, Chao D, Chen ER. Acquired immunity in rats against *Angiostrongylus cantonensis* infection. *Int J Parasitol* 1989;19:617–20.
- Eamsobhana P, Yong HS. Immunological diagnosis of human angiostrongyliasis due to *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae). *Int J Infect Dis* 2009;13:425–31.
- Urban Jr JF, Madden KB, Svetic A, Cheever A, Trotta PP, Gause WC, et al. The importance of Th2 cytokines in protective immunity to nematodes. *Immunol Rev* 1992;127:205–20.
- Uakhowithchai V, Techasoponmani R, Sirisinha S. Effect of immunization with metabolic antigens on experimental infection of rats with *Angiostrongylus cantonensis*. *Southeast Asian J Trop Med Public Health* 1977;8:486–93.
- Morassutti AL, Levert K, Pinto PM, da Silva AJ, Wilkins P, Graeff-Teixeira C. Characterization of *Angiostrongylus cantonensis* excretory–secretory proteins as potential diagnostic targets. *Exp Parasitol* 2012;130:26–31.
- Lee JD, Chen ER, Chung LY, Yen CM. Humoral immune responses in mice infected with gamma-irradiated third-stage larvae of *Angiostrongylus cantonensis*. *Kaohsiung J Med Sci* 1997;13:277–85.
- Yoshimura K, Aiba H, Oya H. Transplantation of young adult *Angiostrongylus cantonensis* into the rat pulmonary vessels and its application to the assessment of acquired resistance. *Int J Parasitol* 1979;9:97–103.
- Ishida K, Yoshimura K. Eosinophil responses of permissive and nonpermissive hosts to the young adult worms of *Angiostrongylus cantonensis*. *Z Parasitenkd* 1986;72:661–71.
- Yoshimura K, Sugaya H, Ishida K. The role of eosinophils in *Angiostrongylus cantonensis* infection. *Parasitol Today* 1994;10:231–3.
- Yong WK, Dobson C. Antibody responses in rats infected with *Angiostrongylus cantonensis* and the passive transfer of protective immunity with immune serum. *Z Parasitenkd* 1982;67:329–36.
- Yong WK, Glanville RJ, Dobson C. The role of the spleen in protective immunity against *Angiostrongylus cantonensis* in rats: splenectomy and passive spleen cell transfers. *Int J Parasitol* 1983;13:165–70.
- Munn EA, Smith TS, Smith H, James FM, Smith FC, Andrews SJ. Vaccination against *Haemonchus contortus* with denatured forms of the protective antigen H11. *Parasite Immunol* 1997;19:243–8.
- Andrews SJ, Hole NJ, Munn EA, Rolph TP. Vaccination of sheep against haemonchosis with H11, a gut membrane-derived protective antigen from the adult parasite: prevention of the periparturient rise and colostral transfer of protective immunity. *Int J Parasitol* 1995;25:839–46.
- Jasmer DP, McGuire TC. Protective immunity to a blood-feeding nematode (*Haemonchus contortus*) induced by parasite gut antigens. *Infect Immun* 1991;59:4412–7.
- Opdebeeck JP, Wong JY, Jackson LA, Dobson C. Hereford cattle immunized and protected against *Boophilus microplus* with soluble and membrane-associated antigens from the midgut of ticks. *Parasite Immunol* 1988;10:405–10.
- Smith SK, Pettit D, Newlands GF, Redmond DL, Skuce PJ, Knox DP, et al. Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of *Haemonchus contortus*. *Parasite Immunol* 1999;21:187–99.
- Smith WD, Smith SK, Murray JM. Protection studies with integral membrane fractions of *Haemonchus contortus*. *Parasite Immunol* 1994;16:231–41.
- Knox DP, Smith SK, Smith WD. Immunization with an affinity purified protein extract from the adult parasite protects lambs against infection with *Haemonchus contortus*. *Parasite Immunol* 1999;21:201–10.
- Smith WD, Skuce PJ, Newlands GF, Smith SK, Pettit D. Aspartyl proteases from the intestinal brush border of *Haemonchus contortus* as protective antigens for sheep. *Parasite Immunol* 2003;25:521–30.
- Knox DP, Redmond DL, Newlands GF, Skuce PJ, Pettit D, Smith WD. The nature and prospects for gut membrane proteins as vaccine candidates for *Haemonchus contortus* and other ruminant trichostrongyloids. *Int J Parasitol* 2003;33:1129–37.
- Maki J, Yanagisawa T. Effect of flubendazole on the number of first-stage larvae of *Angiostrongylus cantonensis* released in the faeces of treated rats. *J Helminthol* 1990;64:87–95.

32. Wang JM, Xu JJ, Zhou YH, Tao JP. [Observation of animal model of life cycle of *Angiostrongylus cantonensis*]. *Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi* 2014;**26**:675–7.
33. Yoshimura K, Sato K, Uchida K, Oya H. The course of *Angiostrongylus cantonensis* infection in athymic nude and neonatally thymectomized mice. *Z Parasitenkd* 1982;**67**:217–26.
34. Liu Z, Wu Y, Feng Y, Wu F, Liu RF, Wang LF, et al. Spleen atrophy related immune system changes attributed to infection of *Angiostrongylus cantonensis* in mouse model. *Parasitol Res* 2017;**116**:577–87.
35. Liu H, Luo X, Shen E, Li H, Ding X, Chen D. Alteration of T cell subtypes in spleen and antibodies of serum in mice infected with *Angiostrongylus cantonensis*. *Parasitol Res* 2013;**112**:1255–60.
36. Chen SN. Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Angiostrongylus cantonensis*. *Trans R Soc Trop Med Hyg* 1986;**80**:398–405.
37. Au AC, Ko RC. Changes in worm burden, haematological and serological response in rats after single and multiple *Angiostrongylus cantonensis* infections. *Z Parasitenkd* 1979;**58**:233–42.
38. Kamiya M, Tanaka H. Hemagglutination test in rats infected with *Angiostrongylus cantonensis*. *Jpn J Exp Med* 1969;**39**:593–9.
39. Newlands GF, Skuce PJ, Knox DP, Smith SK, Smith WD. Cloning and characterization of a beta-galactoside-binding protein (galectin) from the gut of the gastrointestinal nematode parasite *Haemonchus contortus*. *Parasitology* 1999;**119**(Pt 5):483–90.
40. Smith WD, Smith SK. Evaluation of aspects of the protection afforded to sheep immunised with a gut membrane protein of *Haemonchus contortus*. *Res Vet Sci* 1993;**55**:1–9.